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The localization of homozygous deletions has been useful in the identification of new tumor-suppressor genes and in identifying the roles of known genes. Four major approaches can identify such deletions: a candidate gene approach where losses of a particular sequence are sought, a candidate positional search where a genome scanning strategy utilizes spaced markers, a candidate locus approach where markers of a known homozygous deletion of one tumor system are extended to a second tumor type, and an unbiased approach such as the representational difference analysis (RDA). Approaches utilizing RDA, candidate genes, and candidate loci in breast cancer were explored by our group. A homozygous deletion of the DPC4 gene was identified in a breast cancer cell line, suggesting the involvement of this TGF-β-like pathway in this cancer. Recently, we identified in a breast cancer a previously unreported homozygous deletion of the MKK4 gene, involved in stress-activated pathways. This was the first confirmation of MKK4 alterations in breast cancer and suggested that alterations of the MKK4 gene may be among the most common tumor-suppressor abnormalities in breast cancer, seen in 15% of cell lines to date.

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Introduction

A rational approach to the diagnosis and treatment of breast cancer should be enabled through the understanding of precisely how the cancer cells differ from normal cells. The most definitive and most basic means to accomplish this lies in the definition of the genetic mutations which distinguish the DNA of cancer from that of normal cells. Currently, very few tumor-suppressor genes are known to be mutated during the growth of human cancers, and the role of most putative proto-oncogenes has remained unclear. The reasons are rather straightforward. The identification of tumor-suppressor genes has largely depended on the identification of deletions, but most losses and gains of chromosomal material in adult tumors involve very large chromosomal areas, limiting the ability to specify the target gene.

A relatively new technique termed the Representational Difference Analysis (RDA) is especially geared toward identifying genetic deletions, amplifications, and rearrangements regardless of their size. For the first time, a procedure readily allows the definition of the smallest genetic lesions, those that should more rapidly aid the discovery of key genes. We have already shown the utility of this approach, defining a candidate subregion of BRCA2, 24-fold smaller than the initially reported region (1,2). For this IDEA grant, we proposed to explore the use of RDA in breast cancer to identify small homozygous deletions and rearranged DNA fragments.

Additional approaches are now available as well, and the cooperative use of these techniques promises to considerably accelerate the speed of new discovery. One approach is the use of the newly available closely-spaced markers throughout the genome to perform genome-scanning for homozygous deletions, which unlike RDA can be targeted to regions of special interest. Also available is the identification of new candidate genes and signaling pathways that provide markers to test specific hypotheses about potential new tumor-suppressor sites in cancer.

Body

Methods

Procedures of the representational difference analysis (RDA) were performed essentially as described by Wigler and colleagues, as modified by our laboratory (1, 3)(below). PCR assays to detect homozygous deletions were performed as described by us (1, 2, 4). An assay for the presence of the STS (sequence-tagged site) is used to test the original genomic DNAs for the absence of template in the neoplasm; a negative result in the tumor thus is considered to indicate a presumptive homozygous deletion. Confirmation of the homozygous deletion is done by testing of a second nonoverlapping STS.

Briefly, RDA is performed as follows. Amplicons are generated by restriction endonuclease digestion of genomic DNA, followed by the ligation of PCR anchors to each 5' end to serve as primer sites for "whole-genome PCR". When used to clone deleted fragments, the driver amplicon is generated from xenograft or cell line DNA, the tester amplicon from normal DNA. When used to clone sites of rearrangement, the driver amplicon is generated from normal DNA, and the tester amplicon from xenograft or cell line DNA. The amplicons are mixed together, with a vast excess of driver amplicon (500 ng vs. 40 μg) present as a saturated solution (5 μl total). The DNA mixture is melted by boiling and allowed to anneal. Homohybrids of tester DNA are selectively and exponentially amplified by PCR. This constitutes one round of RDA. Subsequent rounds of RDA start with replacement of the anchor primers, and use increasingly smaller ratios of tester to driver amplicon in the hybridization. Two to three rounds can be sufficient to produce an acceptable difference product.

Both RDA analysis and additional candidate loci were used to examine breast cancer DNA samples. The RDA analysis included two breast cancers of patients of the Johns Hopkins

Hospital, propagated as xenografts in nude mice and stored frozen. Normal tissues of each patient were snap frozen, and served as control normal tissue. Twenty-three cell lines available from the ATCC (American Type Culture Collection) were grown in tissue culture. DNA was extracted from xenografts, human tissues, and cell lines using standard techniques and used for the analysis of candidate loci.

Results and Discussion

RDA was initiated on the two xenografts of human breast cancer, but difference products indicative of homozygous deletions were not identified. An opportunity presented itself to rapidly survey for additional homozygous deletions at a novel locus not known at the time of the initial grant application. We had identified a novel tumor-suppressor gene in pancreatic cancer, DPC4, which is at chromosome 18q21.1 (4). A major mechanism of its inactivation was by homozygous loss in tumors. A survey of the 23 ATCC cell lines for sequence changes and homozygous deletions of DPC4 was undertaken. The MDA-MB468 cell line was found to have a homozygous deletion of DPC4 (5). This suggested the involvement of the DPC4 pathway in breast cancer, presumed to involve a TGF-β-like signaling pathway involving cell surface receptors, SMAD proteins (such as DPC4), and the activation of transcription of genes in the nucleus. With this lead, the status of a number of TGF-β superfamily receptors was targeted for homozygous deletion screening, screens for mutations that produce truncated protein products, and sequence analysis. A survey of TGF-β superfamily pathway receptors was negative for homozygous deletions and mutations. Mutational surveys of Smad genes other than DPC4 are negative to date.

Occasional mutations and homozygous deletions of the MKK4 gene in breast cancer cell lines and a few other tumor types were reported by another group, but a survey of additional breast cancers was negative (6). MKK4 is a member of a family of mitogen-activated protein kinases, and initially would appear to be an unusual candidate for a tumor-suppressive role. Yet, cellular stresses are also able to activate the particular pathways in which MKK4 is active, and MKK4 may help to mediate apoptosis and cell differentiation. We explored MKK4 further, identifying and mapping an additional homozygous deletion within a breast cancer cell line. This was the first confirmation of the role of MKK4 in breast cancer (7). The cumulative rate of MKK4 mutations among cell lines studied to date, is 15%. This would make MKK4 one of the most commonly mutated tumor-suppressor genes yet described for breast carcinoma. Our finding suggested that the prior mutational screen probably had been of inadequate sensitivity, since for technical reasons it could not have identified any of the homozygous deletions. Studies are now justified to determine the subsets of breast cancer, if any, in which MKK4 mutations may play the greatest role and whether there are recognizable clinicopathological features associated with the MKK4-mutant tumors.

Homozygous deletion loci, identified from studies of other tumor types but where the gene target had not yet been specified, were previously successful in identifying the genetic alterations of the PTEN gene in breast carcinoma, and aided the cloning of the gene. We have therefore extended our studies of breast cancer to include high resolution genome scanning of candidate loci at such sites identified from other tumor systems. These surveys are negative to date. The possibility of β-catenin mutations was suggested by the known involvement of E-cadherin mutations in lobular carcinoma, but we found no mutations.

Conclusions

Novel tumor-suppressor genes continue to be identified in breast cancer through the study of homozygous deletions. This approach, to identify homozygous deletions in breast cancer and to screen breast cancers for candidate sites of homozygous deletions, appears to be one of the more efficient means to discover novel and important regulatory systems that are impaired in human breast cancers.

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Advances in Brief

DPC4 Gene in Various Tumor Types[1]

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Abstract

We recently identified a novel tumor-suppressor gene, *DPC4*, at chromosome 18q21.1 and found that both alleles of *DPC4* were inactivated in nearly one-half of the pancreatic carcinomas. Here, we analyzed 338 tumors, originating from 12 distinct anatomic sites, for alterations in the *DPC4* gene. Sixty-four specimens were selected for the presence of the allelic loss of 18q and were further analyzed for *DPC4* sequence alterations. An alteration of the *DPC4* gene sequence was identified in one of eight breast carcinomas and one of eight ovarian carcinomas. These results indicate that whereas *DPC4* inactivation is prevalent in pancreatic carcinoma (48%), it is distinctly uncommon (<10%) in the other tumor types examined. The tissue restriction of alterations in *DPC4*, as in many other tumor-suppressor genes, emphasizes the complexity of rate-limiting checkpoints in human tumorigenesis.

Introduction

Allelotype analysis of pancreatic carcinoma has indicated that about 90% of these tumors show allelic loss of chromosome 18q (1). We recently identified the *DPC4* gene (for deleted in pancreatic carcinoma, locus *4*) as a genetic target of these losses (2). *DPC4* was homozygously deleted in about 30% of pancreatic carcinomas and inactivated by intragenic mutation in another 20% of the tumors.

A variety of tumor types exhibit allelic loss of 18q. To survey the involvement of *DPC4* in different tumor types, we analyzed 338 tumors from outside of the gastrointestinal tract for *DPC4* gene alterations. Sixty-four specimens were selected for 18q loss and high neoplastic cellularity and were further analyzed for alterations in the *DPC4* gene sequence.

Materials and Methods

Tumor Samples. Seventy-three of 347 tumor samples were selected for allelic loss of chromosome 18q21 and high neoplastic cellularity. All selected tumor samples are listed in Table 1. The tumor set included bladder, breast, head and neck, hepatocellular, lung, ovarian, prostatic, and renal cell carcinomas, glioblastomas and medulloblastomas, melanomas and osteosarcomas, and nine additional pancreatic carcinomas. The six lung carcinomas included one carcinoid, three small cell lung carcinomas, and two non-small cell lung carcinomas; all three primary ovarian carcinomas were serous carcinomas. Fourty-one of the specimens were primary tumors; 24 were tumor cell lines; and 8 were xenografts.

PCR and Sequencing. Microsatellite analysis and PCR were performed in microtiter plates as described (1, 3). PCR reactions were incubated with 10 units of Exonuclease I and 2 units of shrimp alkaline phosphatase (United States Biochemical Corp., Cleveland, OH) in a final volume of 50 μl PCR buffer for 15 min at 37°C and 15 min at 80°C. Sequencing of 5 μl enzyme-treated PCR product was performed in microtiter plates by Sequitherm cycle sequencing, according the recommendations of the manufacturer (Epicentre Technologies, Madison, WI). PCR and sequencing primers are available on the Internet (http://www.med.jhu.edu/pancreas/index.htm).

Results and Discussion

Sixty-four cancers from outside of the gastrointestinal tract and nine pancreatic carcinomas were analyzed for *DPC4* gene alterations. The tumors were selected from a series of 347 neoplasms for the presence of allelic loss of 18q, as determined by microsatellite analysis using the markers *D18S46, D18S363,* and *D18S474* (Table 2; Ref. 4). True LOH[3] had been determined for the bladder, head and neck, and prostatic carcinomas as part of previous studies by comparison of tumor DNA with constitutional normal DNA. The other specimens were selected on the basis of statistical evidence for LOH, as determined by the presence of a single allele size at each of the three loci in the tumor DNA. With a heterozygosity value of >0.7 for each marker, this selection reflects presumptive LOH, with an estimated $P < 0.03$. Finally, only the tumor samples that had high neoplastic cellularity, as judged by a decrease in allele intensity of at least 50% in the microsatellite analysis, were selected for *DPC4* sequence analysis.

The 11 exons of *DPC4* were amplified by PCR and sequenced directly by cycle sequencing. The breast carcinoma cell line MDA-MB468 was found to have a homozygous deletion of the complete coding sequence of *DPC4,* whereas the flanking microsatellite markers *D18S46, D18S363,* and *D18S474* were retained. The pancreatic carcinoma cell line Colo357 had a homozygous deletion involving exons 1–4 of *DPC4*, whereas the remaining exons were retained. Duplex PCRs for exons 1 and 10 of *DPC4* and the *DPC1* locus at 13q (3) confirmed both homozygous deletions and ensured DNA quality (Fig. 1*A*). Sequence analysis of *DPC4* revealed alterations in the ovarian carcinoma cell line SW626, the pancreatic carcinoma cell lines AsPc1 and Capan1, and the pancreatic carcinoma xenograft MX36 (Fig. 1*B* and Table 3). The alterations in SW626 and AsPc1 predicted nonconservative amino acid replacements (Asp → His and Arg → Thr, respectively), whereas the alterations in Capan1 and MX36 predicted truncations of the protein (a nonsense codon and a 2-bp frameshift, respectively). The mutations were confirmed by sequencing of a second independently amplified PCR product. The constitutional normal DNAs for the tumors with mutations were not available to determine whether the alterations were somatically acquired or present in the germline. Analysis of more than 100 chromosomes, however, had not identified these sequence alterations, rendering them unlikely to be common sequence polymorphisms.

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[3] The abbreviation used is: LOH, loss of heterozygosity.

Table 1 *Tumor samples analyzed for DPC4 gene alterations*

The tumor set selected for *DPC4* sequencing was based on the presence of allelic loss at 18q21 and high neoplastic cellularity.

Tumor type	Sample	Source[a]	Origin[b]
Bladder carcinoma	BT3	P	A
Bladder carcinoma	BT4	P	A
Bladder carcinoma	BT6	P	A
Bladder carcinoma	BT7	P	A
Bladder carcinoma	BT8	P	A
Bladder carcinoma	BT10	P	A
Bladder carcinoma	MX19	X	A
Breast carcinoma	BT483	L	ATCC
Breast carcinoma	BT549	L	ATCC
Breast carcinoma	MCF7	L	ATCC
Breast carcinoma	MDA-MB415	L	ATCC
Breast carcinoma	MDA-MB436	L	ATCC
Breast carcinoma	MDA-MB468	L	ATCC
Breast carcinoma	T47D	L	ATCC
Breast carcinoma	ZR75–30	L	ATCC
Glioblastoma	BX271	X	B
Glioblastoma	BX368	X	B
H&N carcinoma[c]	38T	P	A
H&N carcinoma	225T	P	A
H&N carcinoma	243T	P	A
H&N carcinoma	MX32	X	C
H&N carcinoma	MX47	X	C
Hepatocellular carcinoma	L3	P	D
Hepatocellular carcinoma	L9	P	D
Hepatocellular carcinoma	L10	P	D
Hepatocellular carcinoma	L14	P	D
Hepatocellular carcinoma	L16	P	D
Hepatocellular carcinoma	L18	P	D
Lung carcinoma	H157	P	E
Lung carcinoma	H249	P	E
Lung carcinoma	H727	P	E
Lung carcinoma	N417	P	E
Lung carcinoma	OH1	P	E
Lung carcinoma	MX44	X	C
Medulloblastoma	BX341	X	B
Melanoma	HM + 86	P	F
Melanoma	M91–054	P	F
Melanoma	UACC827	P	F
Melanoma	UACC1022	P	F
Osteosarcoma	Os1	P	D
Osteosarcoma	Os6	P	D
Osteosarcoma	Os7	P	D
Ovarian carcinoma	SO3T	P	G
Ovarian carcinoma	SO6T	P	G
Ovarian carcinoma	SO9T	P	G
Ovarian carcinoma	CaOv3	L	ATCC
Ovarian carcinoma	CaOv4	L	ATCC
Ovarian carcinoma	NIH-OvCar3	L	ATCC
Ovarian carcinoma	SKOV3	L	ATCC
Ovarian carcinoma	SW626	L	ATCC
Pancreatic carcinoma	AsPc1	L	ATCC
Pancreatic carcinoma	Capan1	L	ATCC
Pancreatic carcinoma	Capan2	L	ATCC
Pancreatic carcinoma	Colo357	L	ECACC
Pancreatic carcinoma	Miapaca2	L	ATCC
Pancreatic carcinoma	Panc1	L	ATCC
Pancreatic carcinoma	PL45	L	C
Pancreatic carcinoma	Su8686	L	ATCC
Pancreatic carcinoma	MX36	X	C
Prostatic carcinoma	13T	P	H
Prostatic carcinoma	25T	P	H
Prostatic carcinoma	47T	P	H
Prostatic carcinoma	51T	P	H
Prostatic carcinoma	128T	P	H
Prostatic carcinoma	142T	P	H
Prostatic carcinoma	402T	P	H
Prostatic carcinoma	412T	P	H
Prostatic carcinoma	DU145	L	ATCC
Prostatic carcinoma	LNCaP	L	ATCC
Prostatic carcinoma	PC3	L	ATCC
Renal cell carcinoma	K2	P	D
Renal cell carcinoma	K3	P	D
Renal cell carcinoma	K5	P	D

[a] P, primary tumor; L, cell line; X, xenograft.

[b] Tumor samples were derived from ATCC, American Type Culture Collection; or ECACC, European Collection of Animal Cell Cultures; or obtained from sample banks of: A, David Sidransky; B, Bert Vogelstein; C, Scott E. Kern; D, Ralph H. Hruban; E, Robert A. Cassero, Jr.; F, Paul S. Meltzer; G, Lora Hedrick and Kathleen R. Cho; and H, G. Steven Bova and William B. Isaacs.

[c] H&N, head and neck.

Table 2 *Allelic loss of 18q in various tumor types*

Tumor type	18q loss[a] (this study) *n* %	18q loss[b] (literature) % (Ref.)	No. selected for sequencing[c]
Bladder transitional cell carcinoma	10/83 12	12 (5), 35 (6)	7
Breast carcinoma	8/22 36	8 (7), 24 (8), 31 (9), 35 (10)	8
Glioblastoma	2/20 10		2
Head/neck squamous cell carcinoma	14/50 28	23 (11), <5 (12), 8 (13), 25 (14),31 (15)	5
Hepatocellular carcinoma	6/25 24	9 (16)	6
Lung carcinoma	6/17 35	24 (17), 65 (18), 14 (19)	6
Medulloblastoma	1/10 10		1
Melanoma	4/18 22	22 (20)	4
Osteosarcoma	3/13 23	64 (21), 18 (22)	3
Ovarian carcinoma	8/12 67	47 (23), 29 (24), 27 (25)	8
Pancreatic carcinoma	9/9 100	89 (1)	9
Prostatic carcinoma	14/46 30	45 (26), 26 (27), 19 (28)	11
Renal cell carcinoma	3/22 14	<5 (29), <5 (30)	3
Total			73

[a] Percentages reflect true LOH or presumptive LOH (see text).

[b] Data were derived from the indicated references.

[c] Number of tumor samples after selection for 18q21 allelic loss and high neoplastic cellularity. Some primary tumors that scored as having LOH did not meet the requirements for sequencing, for technical reasons. The tumors are listed individually in Table 1.

Three of the sequence alterations identified in this series were in exon 8, within 25 bp of each other, and one was in exon 2 (Table 3). Although data are limited, the locations of the *DPC4* sequence changes suggest mutational hotspots in exons 8 and 11; 4 of the 11 currently known sequence alterations are in exon 8, and another 4 are in exon 11 (2; this study).[4] Of note, the regions of strongest homology between *DPC4* and the *D. melanogaster Mad* and *C. elegans Sma2* genes include these putative mutational hotspots (2).

We previously reported that *DPC4* was inactivated in 20 of 41 pancreatic carcinoma xenografts (2). These inactivations included 14 homozygous deletions and six intragenic alterations. The identification here of one homozygous deletion and three intragenic alterations in nine pancreatic carcinoma cell lines further substantiates the mutational involvement of *DPC4* in pancreatic carcinoma. Together, 24 (48%) of 50 pancreatic carcinomas examined have been found to have mutational inactivations of *DPC4*.

We previously reported a homozygous deletion in one of two bladder carcinoma xenografts (2). Here, we sequenced the second xenograft and six primary bladder carcinomas but did not identify additional alterations in *DPC4*. It should be noted that the detection of homozygous deletions in primary tumors by standard PCR is generally hampered by the presence of nonneoplastic cells (31). Forty-one of the 73 tumors analyzed here were primary tumors (Table 1), potentially impairing the detection of homozygous deletions in these specimens.

Our data indicated that *DPC4* gene alterations are restricted to tumors arising in specific types of tissue. Many of the tumor types examined exhibit rather low frequencies of 18q LOH, and the two *DPC4* alterations identified in nonpancreatic tumors were in cancers that exhibit moderate or high LOH of 18q (Table 2). However, all tumors tested were selected for 18q LOH; yet, only two alterations were identified in 64 tumors arising outside the gastrointestinal tract. This suggests that other tumor-suppressor gene(s) might be targets of the 18q losses. Analysis of the candidate tumor-suppressor gene *DCC* at 18q has been difficult, due to its size and complexity (32).

Allelotype analyses have suggested that frequent alterations of a rather restricted set of tumor-suppressor genes are likely to be of

[4] A. T. M. S. Hoque and S. E. Kern. Mutational involvement of DPC4 in colitis-associated neoplasia, submitted for publication.

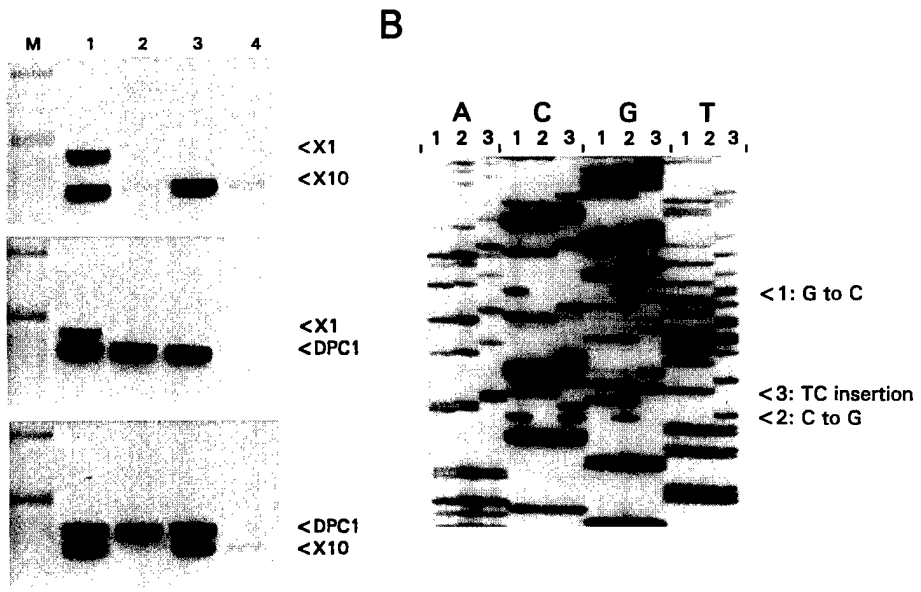

Fig. 1. *A,* Duplex PCR analysis of homozygous deletions involving the *DPC4* gene. *Top panel,* duplex PCR for exons 1 and 10 of *DPC4*; *middle panel,* PCR for exon 1 of *DPC4* and the *DPC1* locus at 13q; *bottom panel,* PCR for exon 10 of *DPC4* and *DPC1*. *M,* 1-kb ladder (Life Technologies, Inc.); *Lanes: 1,* normal DNA serving as a positive control; *2,* breast carcinoma cell line MDA-MB468, which had a homozygous deletion involving the complete coding sequence of *DPC4; 3,* pancreatic carcinoma cell line Colo357, which had a homozygous deletion involving exons 1–4 of *DPC4; 4,* template-negative control. *B,* sequence analysis of mutations in exon 8 of the *DPC4* gene. *Lanes: 1,* ovarian carcinoma cell line SW626, a GAT → CAT missense mutation; *2,* pancreatic carcinoma cell line Capan1, a TCA → TGA nonsense mutation; *3,* pancreatic carcinoma xenograft MX36, a TC insertion.

Table 3 *DPC4 alterations*

Tumor	Tissue	Alteration	Codon	Exon	Predicted effect
MDA-MB468	Breast	homozygous deletion		1–11	No protein
SW626	Ovarian	GAT → CAT	351	8	Asp → His
Colo357	Pancreas	homozygous deletion		1–4	No protein
AsPc1	Pancreas	AGG → ACG	100	2	Arg → Thr
Capan1	Pancreas	TCA → TGA	343	8	Ser → Stop
MX36	Pancreas	TCA → TCTCA	343	8	Frameshift

DPC4 alterations identified in this study in a set of 73 tumor samples. Early studies had identified genetic inactivation of *DPC4* in nearly one-half of pancreatic carcinoma xenografts, three pancreatic carcinoma cell lines (BxPc3, CFPAC1, and HS766T), two colorectal, one biliary, and one bladder carcinoma, and an ulcerative colitis-associated dysplasia (2).[4]

major importance for most tumor types (1, 5–30). A set of inactivated tumor-suppressor genes appears to be characteristic for a particular tumor type and can be distinctive even for tumors that arise in related anatomical sites. Frequent inactivation of the *APC* gene, for example, is characteristic of colorectal carcinomas (33) but not for pancreatic carcinomas (34–36). Vice-versa, the *p16* gene is frequently inactivated in pancreatic carcinomas (37) but not in colorectal carcinomas (38). Indeed, the broad spectrum of tumors that harbor *p53* alterations might be the exception among tumor-suppressor genes (39). The importance of genes that sustain low-prevalence alterations, however, may as yet be underestimated. Such events may contribute significantly to the genetic variety within a tumor type and, thus, to the complexity of human tumorigenesis. Low-prevalence alterations would become increasingly important if multiple alterations of this type accumulated in individual tumors. Allelotype analyses have indeed suggested that this is likely to be the case (1, 5–30).

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Advances in Brief

Alterations in Pancreatic, Biliary, and Breast Carcinomas Support *MKK4* as a Genetically Targeted Tumor Suppressor Gene[1]

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Abstract

Mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) is a component of a stress and cytokine-induced signal transduction pathway involving MAPK proteins. The MKK4 protein has been implicated in activation of JNK1 and p38 MAPK on phosphorylation by conserved kinase pathways. A recent report on the deletion and mutation of the *MKK4* gene in human pancreatic, lung, breast, testicle, and colorectal cancer cell lines suggests an additional role for *MKK4* in tumor suppression. Both the gene function and the infrequency of mutations might be considered atypical for many human tumor suppressor genes, and constitutional DNA was not previously available to determine whether the reported sequence variants had preceded tumor development. Here, we report that homozygous deletions are detected in 2 of 92 pancreatic adenocarcinomas (2%), 1 of 16 biliary adenocarcinomas (6%), and 1 of 22 breast carcinomas (when combined with reported sequence alterations, 3 of 22 or 14%). In addition, in a panel of 45 pancreatic carcinomas prescreened for loss of heterozygosity, one somatic missense mutation of *MKK4* is observed and confirmed in the primary tumor (2%).

Mapping of the homozygous deletions further indicated *MKK4* to lie at the target of deletion. The finding of a somatic missense mutation in the absence of any other nucleotide polymorphisms or silent nucleotide changes continues to favor *MKK4* as a mutationally targeted tumor suppressor gene. Coexistent mutations of other tumor suppressor genes in *MKK4*-deficient tumors suggest that *MKK4* may participate in a tumor suppressive signaling pathway distinct from *DPC4*, *p16*, *p53*, and *BRCA2*.

Introduction

Tumor development is a gradual process involving the accumulation of gene mutations (1). One allele of a tumor suppressor gene is inactivated through germline transmission or a somatic mutation, and inactivation of the second allele is required for tumorigenesis (2). Biallelic inactivation of a tumor suppressor gene often involves a large chromosomal deletion that manifests as LOH[3] of the region. This accompanies a more subtle change that inactivates the other allele, either by a small intragenic change or by a nested deletion that leads to homozygous deletion of the gene and flanking sequences. The inactivations of the *p53* and *APC* genes often occur in the form of intragenic mutations (3–5). In contrast, homozygous deletions can be a means to inactivate the *p16* (6, 7), *BRCA2* (8), and *DPC4* (9) genes.

The discovery of some tumor suppressor genes has been facilitated by the identification of homozygous deletions in chromosomal regions with high frequencies of LOH. Chromosome 17p13 has been of persistent interest because of its high frequency of LOH in many cancer types (10). The inactivation of *p53* at 17p13 does not account for all cases of LOH, spurring speculation regarding other potential tumor suppressors within the region. Using a marker, D19S969, located approximately 10 cM centromeric of the *p53* locus, a homozygous deletion was identified in a pancreatic cancer (11). The *MKK4* gene was mapped within this homozygous deletion. Additional deletions and sequence variants that would inactivate MKK4 protein function were identified at a low rate (3%) in cancers of the breast, colon, testis, and pancreas (11). Two other candidate genes were also identified within the deleted region. Neither was found to harbor any nucleotide variations upon sequencing (11).

We were interested in studying *MKK4* in part because of its reported mutations in pancreatic cancer (11). LOH of distal 17p affected 90% of our pancreatic cancer series (12), a remarkably high figure even when compared with the *p53* mutation rate of 75% (3). An expanded set of pancreatic and distal biliary carcinomas were available to assess the role that *MKK4* may play in pancreatic tumorigenesis. This tumor panel has been well-studied for other known mutations (13) and, therefore, could enable us to infer pathway relationships if *MKK4* abnormalities were found. Because the previous report did not address whether the nucleotide changes and deletions of *MKK4* were acquired or germline variants (11), we hoped to compare the results from cancer-derived DNA and constitutional DNA at sites of abnormality.

Materials and Methods

Tissue Samples and Cell Lines. Pancreatic and biliary cancers were resected at the Johns Hopkins Hospital. At the time of the surgery, normal duodenal mucosa was frozen and stored at −80°C and cancer xenografts were established and processed as described previously (6). Breast cell lines (BT20, BT474, BT483, BT549, Hs578T, MCF7, DU4475, MDA-MB134-VI, MDA-MB157, MDA-MB175-VII, MDA-MB231, MDA-MB415, MDA-MB361, MDA-MB453, MDA-MB436, MDA-MB468, SKBR3, T47D, UACC812P1, UACC893, ZR75–1, ZR-75–30) were purchased from American Type Culture Collection (Manassas, VA).

Homozygous Deletion Analysis. Genomic DNA samples (40 ng/sample) were screened for homozygous deletions using PCR analysis as described previously (6, 8). The primers used to amplify D19S969A and the *MKK4* exons were identical to those described previously (11). The integrin-β-4 primer sequences are INTB4-A-F 5′-gtgtccgtgtggataaggac-3′ and INTB4-A-R 5′-tccatgaccacgatgctctc-3′. The boundaries of homozygous deletions were determined by using the D17S954, D17S1303, WI-6478, WI-5743, WI-2437, WI-2335, and D17S947 dinucleotide repeat and sequence-tagged site markers (Research Genetics, Huntsville, AL).

LOH and Sequence Analyses. LOH was determined using four polymorphic markers (D17S969, D17S1303, D17S954, and D17S947; Research Genetics). LOH was conclusively present when analysis of the tumor DNA showed the loss of one allele in comparison with its corresponding normal DNA. When a normal DNA sample was unavailable, the LOH status was presumptively shown by the unambiguous presence of only a single allele size among all polymorphic markers evaluated. Forty-five samples were randomly selected for sequencing from the tumors having conclusive LOH. Each exon

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[3] The abbreviations used are: LOH, loss of heterozygosity; MKK4, mitogen-activated protein kinase kinase 4; MAPK, mitogen-activated protein kinase.

was amplified by PCR, treated with exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, OH), and subjected to cycle-sequencing (Thermo-Sequenase, Amersham, Arlington Heights, IL). The sequencing primers were taken from genomic sequences of the previous report (11).

Results

Homozygous Deletions Detected in Pancreatic and Biliary Xenografts and a Breast Cancer Cell Line. Using the D17S969A marker and the primers specific for exon C of *MKK4* (CG2exC.FA and CG2exC.RB), we screened a panel of 92 pancreatic ductal adenocarcinomas, 16 distal common bile duct adenocarcinomas, and 19 other carcinomas of the region (predominantly duodenal and ampullary cancers) for homozygous deletions. Twenty-two breast cancer cell lines were also analyzed. Two pancreatic adenocarcinoma xenografts (PX91 and PX359), one biliary tumor xenograft (PX109), and one breast cancer cell line (DU4475) exhibited homozygous deletions at D17S969A and/or *MKK4* exon C (Fig. 1*A* and data not shown). Homozygous deletions were confirmed with duplex PCR, wherein a deleted marker fails to amplify in the same PCR reaction that allows amplification of a second nondeleted marker (Fig. 1*A* and data not shown; 6, 9). Of the 22 breast cancer cell lines examined for homozygous deletions, 2 have been reported previously to harbor sequence mutations (11), bringing the current and reported mutation rate of *MKK4* to 14% in this breast cancer series. Pancreatic cancer PX359 had a homozygous deletion at D17S969A, but not at *MKK4* exon C (Fig 1*A* and Table 1). DU4475 harbored a homozygous deletion at *MKK4* exon C, but not at D17S969A (Table 1). Further analyses revealed that the exon A of *MKK4* (the exon nearest to D17S969) was deleted from PX359 (Fig. 1*B*), but all other exons were present in the tumor. All other homozygous deletions spanned the entire exonic sequence of *MKK4* (Fig 1*B* and Table 1).

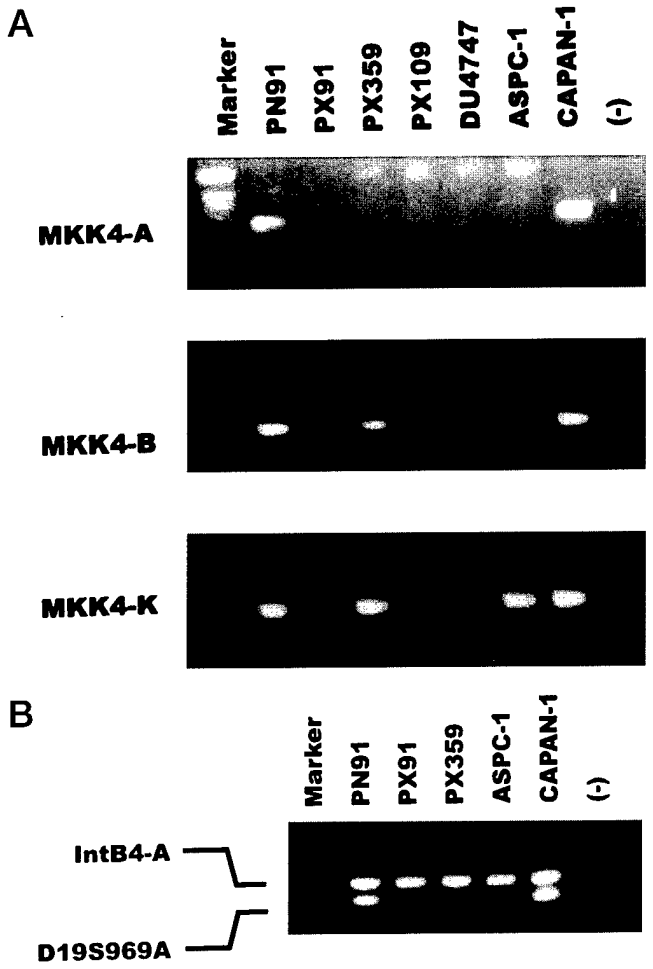

Fig. 1. Homozygous deletions in pancreatic, biliary, and breast cancers. ASPC-1 and CAPAN-1 pancreatic cancer cell lines serve as the negative and positive controls; CAPAN-1 has a nonsense mutation in *MKK4*, and ASPC-1 contains a homozygous deletion from D17S969 to *MKK4* exon C (11). *A*, detection of homozygous deletions in the genomic DNAs of pancreatic cancer xenografts, PX91 and PX359, by duplex PCR using primers for D17S969A and integrin-β-4 (internal control). *B*, mapping of homozygous deletions. The presence of each *MKK4* exon was examined by PCR. The entire coding region of *MKK4* was deleted in PX91, PX109 (biliary cancer), and DU4475 (breast cancer). Only exon A of *MKK4* was deleted in PX359.

Neighboring markers (D17S954, D17S1303, WI-6478, WI-5743, WI-2437, WI-2335, and D17S947) were used to further define the boundaries of the detected homozygous deletions. The distance between the most telomeric marker (D17S954) and the most centromeric marker (D17S947) is 10 cM, and all of the deletions were confined exclusively within the region defined by the two inner markers, WI-5743 and D17S947 (Table 1). Sequences at D17S954, D17S303, and WI-6478 were also retained in these cancer samples.

LOH Analysis. We screened pancreatic cancer xenografts for LOH with the highly polymorphic markers D17S969, D17S1303, D17S954, and D17S947. LOH of 17p at the *MKK4* locus was seen in 73 of 83 xenografts (88%). Conclusive LOH was found in 48 of the 55 cases for which normal DNA was available (87%); presumptive LOH was inferred in 25 of the 28 cases wherein normal DNA was not available (89%), as defined in "Materials and Methods." Larger and smaller alleles of each pair were lost at a ratio of 1:1. We also noted evidence of *MKK4* as a target of allelic loss. PX359 had two alleles at marker D17S954, the most telomeric marker. DU4475 had two alleles at D17S954, D17S1303, and WI-6478, the three most telomeric markers. Thus, the LOH accompanying the homozygous deletion of these tumors did not extend into the (more telomeric) p53 locus.

A Missense Mutation Detected in a Pancreatic Cancer. We examined the exonic sequences and splice junctions of the *MKK4* gene in 45 xenografts exhibiting conclusive LOH. One mutation was discovered at codon 12 within exon A, creating a missense mutation from glycine to serine in tumor sample PX17 (Table 1). The corresponding normal sample for PX17 did not harbor the same nucleotide change, indicating a somatic mutation origin, presumably during tumor development. DNA of the corresponding primary cancer tissue was prepared using cryostat-dissection, sequenced, and the mutation was verified. Two tumor samples were found to share the same polymorphism (T to C) in the 5′-untranslated region of exon A, 82 bp upstream of the ATG start site (data not shown). Due to the technical difficulty of sequencing a G-C rich domain, we were able to examine exon A sequences in only 36 of 45 xenografts (80%). All other exons were sequenced in entirety, and no other mutation was identified.

Clinicopathological Review. Review of the histological features of the original resected tissues and of the history and clinical courses of the four patients revealed no distinctive features among the pancreatic and biliary cancers harboring *MKK4* gene inactivation. All four patients had a history of tobacco smoking.

Discussion

MKK4 belongs to the MAPK kinase family and has been shown to specifically phosphorylate JNK1 and p38, but not ERK1 (14, 15). C-jun and the closely related jun family members are the known substrates for JNK1 (16). MKK4 protein can be activated by MEKK, which is part of the Ras-dependent and cytokine/stress-induced signaling cascades (14, 15). Given the proto-oncogene Ras or Ras-like proteins upstream and C-jun downstream, MKK4 holds an unusual pathway relationship for a tumor suppressor gene. The role that

Table 1 *MKK4 genetic alterations in pancreatic, biliary, and breast cancers*[a]

Sample	Carcinoma type	Sequence alteration[b]	D17S954 D17S1303 WI6478, WI5743	D17S969	*MKK4* Exon A	*MKK4* Exons B–K	WI2437	WI2335	D17S947
PX17	Pancreatic	Codon 12 GGC to AGC	Ret.	Ret.	Ret.	Ret.	Ret.	Ret.	Ret.
PX91	Pancreatic	N.A.	Ret.	H.D.	H.D.	H.D.	Ret.	Ret.	Ret.
PX359	Pancreatic	N.A.	Ret.	H.D.	H.D.	Ret.	Ret.	Ret.	Ret.
PX109	Biliary	N.A.	Ret.	H.D.	H.D.	H.D.	H.D.	H.D.	Ret.
DU4475	Breast	N.A.	Ret.	Ret.	H.D.	H.D.	H.D.	Ret.	Ret.

[a] Ret., one allele retained; H.D., homozygous deletion. All markers and exons are placed in chromosomal map order, from telomeric (left) to centromeric (right).
[b] N.A., not applicable due to homozygous deletion.

MKK4 plays in the known stress-induced pathway may indeed prove to be important for tumor suppression (11). However, it is also possible that MKK4 possesses other uncharacterized biological functions *in vivo*.

Nonetheless, genetic evidence strongly suggests that *MKK4* is a candidate tumor suppressor gene. Homozygous deletions of *MKK4* were observed previously in one pancreatic and one lung cancer cell line (11). In addition, sequence variants of *MKK4* were detected in two cell lines of breast cancer and one each of pancreatic, colorectal, and testicular cancers, resulting in a 3% total frequency of genetic alteration in the examined cell lines (11). Here, we report homozygous deletions in two pancreatic (2%) and one biliary (6%) tumor xenografts. A somatically acquired missense mutation of *MKK4* was detected in a pancreatic cancer (2%). A homozygous deletion of *MKK4* was observed in one breast cancer cell line. When combined with the results of cell lines in the previous report, the total frequency of genetic alteration of *MKK4* in the 22 breast cancer cell lines is at least 14%. The combined homozygous deletion map derived from the two studies indicates a consensus, with breakpoints within or near *MKK4*, strongly suggesting that *MKK4* represents the target gene of the deletions. Indeed, the minimal consensus deletion is defined by the span of the *MKK4* gene itself, as the deletions do not all overlap a particular marker. A similar pattern of deletion was originally used to justify the tumor suppressor candidate, *DPC4* (9). Two other genes cloned from a homozygous deleted region between *MKK4* and D19S969A were shown to lack any sequence variants in 89 miscellaneous cancer cell lines (11), and these genes lie outside the minimal consensus of deletion. Together, both groups have found no silent mutations of *MKK4*. We also found evidence, in tumors having an *MKK4* genetic alternation, that *MKK4* can be an independent target for LOH; the LOH contributing to *MKK4* inactivation is not merely a byproduct of the large deletions that target the nearby p53 gene.

In addition to the purely genetic data, nearly all mutated *MKK4* sequences were shown to code for truncated or altered proteins that do not exhibit normal kinase activity (11). Furthermore, there are two potential start sites of translation for the MKK4 protein, and the location of our missense mutation at codon 12 might be seen to favor one of them as the biological start site of translation. Only the 5′ start site would produce a protein that incorporates the mutation identified.

It might seem puzzling that the mutational frequency of *MKK4* is relatively low, but there are many examples of previously reported tumor suppressor genes with low mutation frequency. SMAD2 has a low mutation frequency in colorectal cancer (17). DPC4 is inactivated frequently among pancreatic cancers (9, 18), but only at a low rate in other cancer types (19–21). The incidence of somatic mutations of BRCA2 is extremely low in many cancer types examined (22–26). In the absence of a mismatch repair deficiency, the transforming growth factor-β type II receptor has a low mutation rate, thus far reported only in colorectal and head/neck cancers (27, 28).

Even at low mutational rates, the discovery of each new tumor suppressor gene aids the recognition of new regulatory pathways or facilitates the progressive elaboration of known suppressive pathways, member by member. For example, based on the presence of coexistent mutations in the same tumor samples, the *MKK4* suppressive pathway would be predicted to be distinct from the *p53*, *p16*, *DPC4*, and *BRCA2* pathways. Genetic inactivation of the *p53*, *p16*, and *DPC4* genes are known to coexist in PX91, ASPC-1, and CAPAN-1 (6, 9, 13). In addition, *BRCA2* is mutated in CAPAN-1 (24).

Homozygous deletion appears to be a common mechanism for inactivation of tumor suppressor genes in pancreatic cancer. Over 60% of pancreatic cancers harbor such deletions, and nearly 20% have at least two (13). Previously, the *DPC4*, *BRCA2*, and *p16* genes were found to be inactivated via homozygous deletion in pancreatic tumors (6, 8, 9, 18). *MKK4* is the fourth independent site of homozygous deletion characterized in pancreatic cancer, and the third site to aid the isolation of a novel suppressor gene (8, 9). Additional efforts to identify and map the homozygously deleted regions in pancreatic tumors should further facilitate the isolation of additional tumor suppressor genes.

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Genetic Alterations of the Transforming Growth Factor β Receptor Genes in Pancreatic and Biliary Adenocarcinomas[1]

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ABSTRACT

Transforming growth factor β (TGF-β) is an extracellular ligand that binds to a heterodimeric receptor, initiating signals that regulate growth, differentiation, and apoptosis. Many cancers, including pancreatic cancer, harbor defects in TGF-β signaling and are resistant to TGF-β-mediated growth suppression. Genetic alterations of *DPC4*, which encodes a DNA binding protein that is a downstream component of the pathway, most frequently occur in pancreatic and biliary carcinomas. We searched for other targets of mutation of the TGF-β pathway in these cancers. We report somatic alterations of the TGF-β type I receptor gene *ALK-5*. Homozygous deletions of *ALK-5* were identified in 1 of 97 pancreatic and 1 of 12 biliary adenocarcinomas. A germ-line variant of *ALK-5*, presumably a polymorphism, was identified, but no somatic intragenic mutations were identified upon sequencing of all coding regions of *ALK-5*. Somatic alterations of the TGF-β type II receptor gene (*TGFBR2*) were identified in 4 of 97 (4.1%) pancreas cancers, including a homozygous deletion in a replication error-negative cancer and three homozygous frameshift mutations of the poly(A) tract of the TGF-β type II receptor in replication error-positive cancers. We also studied other related type I receptors of the TGF-β superfamily. In a panel of pancreas cancers preselected for loss of heterozygosity at the *ALK-1* locus, sequencing of all coding exons of the *ALK-1* gene revealed no alterations. No homozygous deletions were detected in the *ALK-1*, *ALK-2*, *ALK-3*, or *ALK-6* genes in a panel of 86 pancreatic cancer xenografts and 11 pancreatic cancer and 22 breast cancer cell lines. The rate of genetic inactivation of TGF-β pathway members was determined in 45 pancreatic cancers. Eighty-two % of these pancreatic cancers had genetic inactivation of the *DPC4*, *p15*, *ALK-5*, or *TGFBR2* genes. Our results indicate that the TGF-β type I and type II receptor genes are selective targets of genetic inactivation in pancreatic and biliary cancers.

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Journal of Negative Observations in Genetic Oncology

NOGO

"Normal Beta-Catenin Gene in Breast Cancer"

M Goggins; W Hilgers; SE Kern
Submitted 07/29/97

Gene	Tumor Type	Number of Cases
Beta-catenin	Breast	23

Exon 3 of the beta-catenin gene, containing the N-terminal oncogenic domain, was amplified by PCR from genomic DNA of 2 xenografts and 21 ATCC cell lines of breast carcinoma. PCR cycle sequencing was performed, and no mutations were found.

Addendum:

Specimen type: 21 cell lines, 2 xenografts, 0 primary tumors, 0 metastatic tumors, 0 constitutional DNA

Methods: Sequencing for mutations

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Journal of Negative Observations in Genetic Oncology

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"Absence of ALK-5 mutations or deletions in breast cancer cell lines"

M Goggins; M Shekher; K Turnacioglu; CJ Yeo; RH Hruban; SE Kern
Submitted 08/04/98

Gene	Tumor Type	Number of Cases
ALK5 (TGFBR1)	Breast	22

The ALK-5 gene was screened as a mutational target in breast carcinoma. A homozygous deletion search was performed using primers that amplified the ALK5 gene. No homozygous deletions were detected. LOH at the ALK-5 region was presumptively determined using the microsatellite markers D9S272, D9S154, D9S258 and D9S1782 in 22 breast cancer cell lines and 2 breast carcinoma xenografts. Upon genomic sequencing of all coding exons of the 6 breast cancer cell lines and 2 xenografts with LOH at 9q, no ALK-5 mutations were detected. Resistance to TGF beta-mediated growth suppression is a common phenomenon in breast cancer cell lines. Previous studies have demonstrated that DPC4 and TGF-beta type II receptor mutations occur very rarely in breast carcinoma. Loss of expression of the TGF beta receptors has been described in a proportion of cancers and could be important. Putative common genetic targets of inactivation within the TGF beta pathway in breast cancers remain to be determined.

Addendum:

Specimen type: 22 cell lines, 0 xenografts, 0 primary tumors, 0 metastatic tumors, 0 constitutional DNA

Methods: PCR for homozygous deletions
Sequencing for mutations

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